PI 1191543

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

July 13, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/459,010*
FILING DATE: *March 31, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/09954*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. Tarver

M. TARVER
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

03/31/03 J1049 U.S. PTO

24-1-03 60459010.033

PROVISIONAL PATENT APPLICATION TRANSMITTAL AND COVER SHEET

[Under 37 CFR 1.53(c)]

Our Docket No.: 14639	Date: March 31, 2003
First Named Inventor: Markus Stoffel	
Title: **Method for Identifying Agents that Mediate the Phosphorylation of Foxa-2**	
Express Mail No.: EV 040988826US	

03/31/03 J1036 U.S. PTO 60/459010

ADDRESS TO: **Box Provisional Application**
Commissioner for Patents
Washington, D.C. 20231

APPLICATION ELEMENTS

1. ☒ Fee Calculation Sheet
   (Submit an original and a duplicate for fee processing)
2. ☒ Applicant claims small entity status
3. ☒ Specification Total Pages: **20**
   - ☒ Descriptive title of the invention
   - ☐ Cross References to Related Applications
   - ☒ Statement Regarding Fed. Sponsored R&D
   - ☐ Reference to Microfiche Appendix
   - ☒ Background of the Invention
   - ☒ Brief Summary of the Invention
   - ☒ Brief Description of the Drawings (if filed)
   - ☒ Detailed Description
   - ☐ Claims
   - ☒ Abstract of the Disclosure
4. ☒ Drawings (35 U.S.C. 113) Total Sheets: **5**
5. ☐ Application Data Sheet (37 C.F.R. 1.76)
6. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission (*If applicable, all necessary*)
   - a. ☐ Computer Readable Form (CRF)
   - b. ☐ Specification Sequence Listing on:
     - i. ☐ CD-ROM or CD-R (2 copies); or
     - ii. ☐ paper
   - c. ☐ Statement verifying identify of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 C.F.R. 3.73(b) Submission
10. ☐ Power of Attorney
11. ☒ Return Receipt Postcard
    (*Should be specifically itemized*)
12. ☐ Other

13. ☒ This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. Name of the agency: National Institutes of Health

INVENTOR(S)

Given Name (First and M.I.)	Family Name	Residence (City and State or Foreign Country)
Markus	Stoffel	New York, NY
Christian	Wolfrum	New York, NY
Daniel	Besser	New York, NY

CORRESPONDENCE ADDRESS

ATTY NAME	Janet M. MacLeod Reg. No. 35,263	SIGNATURE: Janet M MacLeod DATE:March 31, 2003
ADDRESS	DORSEY & WHITNEY LLP 250 Park Avenue New York, New York 10177	Customer Number: 30873
TELEPHONE	212-415-9200	
FAX	212-953-7201	

[redacted]

APPLICATION FEE TRANSMITTAL SHEET (FOR FY 2003)

Complete if Known	
Application No.	
Filing Date	March 31, 2003
First Named Inventor	Markus Stoffel
Group Art Unit	
Examiner Name	
Atty. Docket Number	14639

METHOD OF PAYMENT (Check One)

1. ☐ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:
   Deposit Account No.: 50-2054
   Deposit Account Name: DORSEY & WHITNEY LLP
   ☒ Charge any additional fee required under 37 C.F.R. 1.16 and 1.17
   ☐ Applicant claims small entity status (see 37 C.F.R. 1.27)\
2. ☒ Check Enclosed

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	750	201	375	☒ Utility Filing Fee	
106	330	206	165	☐ Design Filing Fee	
108	750	208	375	☐ Reissue Filing Fee	
114	160	214	80	☒ Prov. Filing Fee	80.00
				Subtotal (1)	**80.00**

2. EXTRA CLAIM FEES

	Number Claims		Prior		Extra		Fee from Below	=	Fee Paid
Total		-	20	=		x	9/18	=	
Indep.		-	3	=		x	42/84	=	
Multiple Dependent Claims						x	140/280	=	
							Subtotal (2)		

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent Claim
109	84	209	42	Reissue independent claims over original patent
110	18	210	9	Reissue claims in excess of 20 and over original patent

FEE CALCULATION (Continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee paid
105	130	205	65	Surcharge - Late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
115	110	215	55	Extension for reply within first month	
116	410	216	205	Extension for reply within second month	
117	930	217	465	Extension for reply within third month	
118	1,450	218	725	Extension for reply within fourth month	
128	1,970	280	985	Extension for reply within fifth month	
120	320	220	160	Filing a brief in support of an appeal	
121	280	270	140	Request for oral hearing	
138	110	248	55	Terminal Disclaimer Fee	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,300	242	650	Utility/Reissue issue fee (inc. advance copies)	
143	470	243	235	Design issue fee (inc. advance copies)	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
195	300	195	300	Publication fee for early, voluntary, or normal publication	
196	300	196	300	Publication fee for republication	
126	180	126	180	Submission of IDS	
581	40	81	40	Recording each patent assignment per property (times number of properties)	
179	750	279	375	Request for Continued Examination (RCE)	
Other fee (specify)					
				Subtotal (3)	
				Total Amount of Payment:	80.00

Name: Janet M. MacLeod	Reg. No.: 35,263	Telephone: (212) 415-9200
Signature: Janet M MacLeod		Date: March 31, 2003

[redacted]

WE, MARKUS STOFFEL, CHRISTIAN WOLFRUM, and DANIEL BESSER, citizens of Germany and residents of the United States, have invented certain new and useful improvement in:

METHOD FOR IDENTIFYING AGENTS THAT MEDIATE THE PHOSPHORYLATION OF FOXA-2

of which the following is a

SPECIFICATION

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

[0001] This invention was made with government support under National Institutes of Health Grant RO1 DK55033. The government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

[0002] The hepatocyte nuclear factor 3 (Hnf-3)/forkhead family of transcription factors in mammals includes three genes designated as Foxa-1 (Hnf-3α), Foxa-2 (Hnf-3β) and Foxa-3 (Hnf-3γ). Kaestner et al. (1994) Genomics 20: 377-385. These factors have in common a highly conserved 100 amino acid winged- helix motif that is responsible for monomeric recognition of specific DNA target sites. Brennan (1993) Cell 74: 773-776. Foxa proteins play a central role in maintaining normal glucose homeostasis by regulating gene expression of rate-limiting enzymes of gluconeogenesis and glycogenolysis in the liver and kidney, including phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pc), and by regulating glucagon and Pdx-1 gene expression in pancreatic α- and β-cells, respectively. O'Brien et al. (1995) Mol. Cell Biol. 15: 1747-1758; Gerrish et al. (2000) J. Biol. Chem. 275: 3485-3492; Lee et al. (2002) Diabetes 51: 2546-2551; Shih

et al. (1999) Proc. Natl. Acad. Sci. USA 96: 10152-10157; Tan et al. (2002) Hepatology 35: 30-39.

[0003] The activity of the gluconeogenic enzyme Pepck is modulated by alterations in the abundance of the protein, principally through transcriptional control of gene expression. In the liver, insulin regulates gene expression of enzymes of gluconeogenesis and glycogenolysis by suppressing transcriptional activity. These pathways ensure that hepatic glucose production is suppressed in the fed state (when insulin levels are increased) and glucose levels are maintained in times of starvation (when serum insulin is low and glucagon is increased). Granner et al. (1983) Nature 305: 549-551. The inability of insulin to suppress endogenous glucose production by inadequate insulin secretion in type 1 diabetes or impaired insulin signaling in type 2 diabetes contributes to the pathogenesis of these common metabolic diseases. Bergman (1997) Recent Prog. Horm. Res. 52: 359-385. The mechanisms by which insulin signaling pathways can modulate the activity of key transcription factors involved in the regulation of metabolic genes is incompletely understood.

[0004] The serine/threonine kinase PKB/Akt is one downstream mediator of phosphatidylinositol 3-kinase (PI3-kinase) and plays an important role in mediating effects of insulin on hepatic glucose production, glycogen and protein synthesis. Franke et al. (1995) Cell 81: 727-736; Franke et al. (1997) Science 275: 665-668; Hardt et al. (2002) Circ. Res. 90: 1055-1063. Upon activation, Akt is translocated to the nucleus where it exerts effects on gene activity by phosphorylation of target proteins like Gsk3, Bad and Fkhrl1. Meier et al. (1999) J. Recept. Signal Transduct. Res. 19: 121-128; Datta et al. (1999) Genes Dev. 13: 2905-2927. Genetic studies of the PI3-kinase–Akt signaling pathway in the nematode *C.elegans* have established that this signaling cascade suppresses the function of the transcription factor daf16, which belongs to the *forkhead*/winged-helix family of transcription factors. Mutations in the Insulin/Igf-1 receptor homologue (*daf-2*), the catalytic subunit of PI3-kinase (*age-1*), or Akt (*akt1* and *akt2*) result in increased longevity and constitutive *dauer* formation, a stage of developmental arrest and reduced metabolic activity that enhances survival

periods of food deprivation and other environmental stresses. Kenyon et al. (1993) Nature 366: 461-464. In each case, mutation of daf-16 restored normal life span and prevented entry into *dauer* stage. Gottlieb et al. (1994) Genetics 137: 107-120; Ogg et al. (1997) Nature 389: 994-999. Subsequently, studies in mammals have shown that the Fkhr (Foxo-1), Fkhrl1 (Foxo-3) and AFX (Foxo-4) genes, members of the human forkead family, also constitute downstream targets of Akt. Biggs et al. (1999) Proc. Natl. Acad. Sci USA 96: 7421-7426; Brunet et al. (1999) Cell 96: 857-868; Kops et al. (1999) Nature 398: 630-634. For instance, the Foxo-1 protein has been shown to be phosphorylated by Akt, which causes repression of transcriptional activity of insulin growth factor binding protein–1 (Igfbp-1), and G6pc. Nakae et al. (2001) J. Clin. Invest. 108: 1359-1367. Furthermore, genetic studies in mice have provided evidence that downstream components of the insulin/Igf-1 signaling pathway are essential for normal energy homeostasis and growth. Mice lacking Akt2 have an impaired ability of insulin to inhibit glucose production in the liver and muscle. Cho et al. (2001) Science 292: 1728-1731. In contrast, mice lacking Akt1 have normal glucose homeostasis, but impaired fetal and postnatal growth. Cho et al. (2001) J. Biol. Chem. 276: 38349-38352.

[0005] In accordance with the present invention, it has been discovered that Foxa-2 is a novel target of Akt and that phosphorylation at a single conserved site is both necessary and sufficient to inhibit the transcriptional activity of Foxa-2.

BRIEF SUMMARY OF THE INVENTION

[0006] The present invention provides a method of identifying agents that mediate the phosphorylation of Foxa-2. Such agents are useful for the treatment of Type 2 diabetes (non-insulin dependent diabetes mellitus).

[0007] The present invention further provides a method for inhibiting gluconeogenesis comprising contacting a cell with an agent that activates the phosphorylation of Foxa-2.

[0008] In another embodiment, the present invention provides a method of treating Type 2 diabetes comprising administering to a subject in need of such treatment a composition comprising an agent that activates the phosphorylation of Foxa-2.

[0009] Agents that mediate the phosphorylation of Foxa-2 and compositions comprising such agents are also provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figs. 1A-D depict the effect of insulin on Foxa-2 activity. Fig. 1A is a bar graph depicting relative Foxa-2 activity in HepG2 cells transfected with an expression vector for Foxa-2 and pPepck-Luc (gray bars) or p6xCdx-TkLuc (black bars). Cells were treated with insulin alone or in the presence of Ly294002 or PD98059. Fig. 1B is a bar graph depicting relative Foxa-2 activity in HepG2 cells transfected with p6xCdx-TkLuc and treated with insulin alone or in the presence of Ly294002 or PD98059. Fig. 1C shows an RT-PCR analysis of Foxa-1-3 and target genes in primary hepatocytes grown in the presence of insulin (50 nM), Ly294002 and PD98059 for 6 hours prior to gene expression analysis. Fig. 1D is a bar graph depicting relative Foxa-2 activity in HepG2 cells transfected with an expression vector for Foxa-1, Foxa-2, Akt or Akt_{K179A} alone or in combination, using p6xCdx-TkLuc as reporter gene. In all experiments luciferase activity was normalized to β-Gal activity. Values are mean of 6 independent experiments ± SD.

[0011] Fig. 2A is a sequence alignment of orthologous and paralogous members of the Foxa family. Fig. 2B is a bar graph depicting relative Foxa-2 activity in HepG2 cells transfected with expression vectors for Foxa-2, Foxa-2_{T156A} or Foxa-

2_{R153K} together with Akt in varying concentrations. p6xCdx-TkLuc was used as the reporter gene. Fig. 2C is a bar graph depicting relative Foxa-2 activity in HepG2 cells transfected with expression vectors for Foxa-2, Foxa-2_{T156A} or Foxa-2_{R153K} together with Akt at the indicated concentrations. pPepck-Luc was used as the reporter gene. In all experiments luciferase activity was normalized to β-Gal activity. Values are mean of 6 independent experiments.

[0012] Fig. 3 is a Western blot of cell lysates (upper panel) and precipitates (lower panel) of HEK/293 cells transfected with expression vectors for Foxa-2, Foxa-2_{T156A} or Foxa-2_{R153K} together with HA-Akt. HA-Akt was precipitated using an HA-antibody; Foxa-2 was precipitated using an anti-Foxa-2 antibody. Cell lysates and precipitates were separated by SDS-PAGE and analyzed for Foxa-2 or Akt by Western blotting.

[0013] Fig. 4 is an autoradiograph demonstrating that Akt can phosphorylate Foxa-2 on residue TI56. Recombinant GST-Foxa-2, GST-Foxa-22_{T156A} or a positive control were incubated with precipitated HA-Akt or HA-LCK-Akt and [γ^{32P}]-ATP. Proteins were separated by SDA-PAGE and phosphorylated proteins were visualized by autoradiography.

[0014] Fig. 5A depicts the results of an electrophoretic mobility shift assay of cell extracts (CE) from insulin-stimulated HEK/293 cells transfected with Foxa-2 or Foxa-2_{T156A} together with Akt. The Foxa-2 binding site of Igfbp-1 was used to shift proteins; a consensus Foxa-2 binding site was used for competition. Supershift was performed using anti Foxa-2 antibody. Fig. 5B depicts untransfected and Akt-transfected HepG2 cells treated with insulin (50 nM), Ly294002 or PD98059 (10 μM), alone or in combination, decorated with anti-Foxa-1 or Foxa-2 antibodies, and visualized with an anti-rabbit IgG-Alexa 480 antibody using laser scanning microscopy. Fig. 5C depicts HepG2 cells transfected with expression vectors for either HA-Foxa-2 or HA-Foxa-2_{T156A} and treated with insulin (50 nM), decorated with an anti-HA antibody, and visualized with an anti-rabbit IgG-Alexa 480 antibody using

laser scanning microscopy. Control cells were starved for 10 hours. All other experiments were performed in medium containing 10% fetal calf serum.

DETAILED DESCRIPTION OF THE INVENTION

[0015] In accordance with the present invention, it has been discovered that Foxa-2, a member of the *forkhead*/winged-helix family of transcription factors, contributes to the regulation of gene expression by insulin and provides a target for mediating effects of insulin and Akt (Protein kinase B) on gene expression. In particular, it has been discovered that Foxa-2 is a novel substrate for Akt phosphorylation and that Foxa-2 transcriptional activity is inhibited by insulin through activation of the phosphatidylinositol-3 kinase (PI3-kinase)–Akt pathway. Threonine phosphorylation of Foxa-2 at a single conserved residue leads to sequestration of Foxa-2 protein in the cytoplasm and through this mechanism is involved in coordinately regulating the expression of metabolic genes and genes that control cellular homeostasis.

[0016] The forkhead transcription factors Foxa-1, -2 and -3 are structurally related proteins that regulate the expression of three key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (Pepck), glucose-6-phosphatase (G6pc) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (F2,6Bpase) by binding to specific sites in their promoters as monomers. Foxa proteins have almost identical DNA binding domains, suggesting redundancy in the transcriptional control of these genes. In accordance with the present invention, it has been discovered that the Akt phosporylation site of Foxa-2 (aa152-157, RRSYTH) is highly conserved among Foxa-2 proteins but absent in Foxa-1 and –3, albeit being located in a region that is otherwise highly conserved between Foxa genes. Furthermore, only Foxa-2 but not Foxa-1 is inactivated by insulin by nuclear exclusion, indicating that Foxa-2 is the principal Foxa protein regulated by insulin. The discovery that Foxa-2 is a novel substrate for insulin mediated Akt regulation implicates this pathway in unrestrained

hepatic gluconeogenesis in type 2 diabetes. Agents that mediate Foxa-2 phosphorylation are thus useful in the treatment of type 2 diabetes.

[0017] In one embodiment, the present invention provides a method of identifying agents that mediate the phosphorylation of Foxa-2. Mediation of phosphorylation includes inhibition and activation of phosphorylation. Agents that inhibit phosphorylation are useful as antihypoglycemic agents. Agents that activate phosphorylation are useful as potential agents in the treatment of diseases that may be ameliorated by inhibiting the transcriptional activity of Foxa-2. Phosphorylation of Foxa-2 leads to nuclear exclusion and inhibition of target genes, including genes of gluconeogenesis and glycogen synthesis. Accordingly, agents that activate phosphorylation of Foxa-2 are useful for the treatment of type 2 diabetes.

[0018] The method of identifying agents that mediate the phosphorylation of Foxa-2 comprises combining a candidate agent with a polypeptide having Akt kinase activity and a substrate comprising the phosphorylation domain of Foxa-2, assaying for phosphorylation of the substrate in the presence and absence of the candidate agent, and comparing phosphorylation in the presence and absence of the candidate agent, whereby a change in phosphorylation of the substrate in the presence of the candidate agent is indicative of the identification of an agent that mediates phosphorylation of Foxa-2.

[0019] The polypeptide having Akt kinase activity may be naturally occurring or synthetic Akt, or fragments or modifications thereof that maintain serine/threonine kinase activity. Mammalian Akt, also known as protein kinase B (PKB), is known in the art and includes isoforms such as Akt 1, Akt 2 and Akt 3. Akt orthologs have also been cloned from other species including D. melanogaster and C. elegans. Datta et al. (1999) Genes Dev. 13: 2905-2927.

[0020] The structure of Akt has been well-characterized and is reviewed by Datta et al. The protein contains a central kinase domain with specificity for serine or threonine residues in the substrate, an amino-terminal domain that mediates lipid-

protein and/or protein-protein interactions, and a carboxy terminus that includes a hydrophobic and protein rich domain. The primary structure is conserved evolutionarily except for the carboxy-terminal tail. Accordingly, one of ordinary skill in the art can determine fragments and modifications of Akt that maintain activity and are useful in the present method.

[0021] The polypeptide having Akt kinase activity can be purified or synthesized by methods known in the art, or obtained commercially. In a preferred embodiment, the polypeptide used in the present invention is human Akt 1 or human Akt 2. Recombinant Akt 1 or Akt 2 is preferred.

[0022] The substrate comprising the phosphorylation domain of Foxa-2 is a peptide or polypeptide comprising a domain having the amino acid sequence RRSYTH. In a preferred embodiment the substrate is a Foxa-2 protein or a fragment or modification thereof comprising the phosphorylation domain. Mammalian Foxa-2 is known in the art. See e.g. Kaestner (2000) TEM 11: 281-283. In a preferred embodiment, the substrate is human Foxa-2. Human Foxa-2 may be purified or synthesized by methods known in the art.

[0023] The method may be performed by providing the polypeptide having Akt kinase activity and a substrate comprising the phosphorylation domain of Foxa-2 in a cell-free *in vitro* system under conditions for phosphorylation. The method may also be performed in a cell extract or cells into which nucleic acids encoding the polypeptide and substrate have been introduced, or in which each naturally occurs, or in which one naturally occurs and the other has been introduced by standard methods of recombinant technology.

[0024] In a preferred embodiment, the method is performed in an *in vitro* system, the polypeptide having Akt kinase activity is mammalian Akt and the substrate is mammalian Foxa-2. Akt and Foxa-2 are preferably recombinantly produced, and in another preferred embodiment are human Akt and human Foxa-2.

[0025] Candidate agents include any chemical compound, and may be naturally occurring or synthetic. Combinatorial libraries of candidate agents may be used. In a preferred embodiment, well-known automated methods of high throughput screening are used to assay candidate agents.

[0026] Phosphorylation of the substrate may be measured by kinase assays known in the art. For example, in a typical *in vitro* kinase assay, the kinase and substrate are incubated in the presence of radiolabeled ATP, e.g. [γ^{32P}]-ATP, in a suitable buffer, e.g. a buffer containing $MgCl_2$ and $MnCl_2$. The substrate is immunoprecipitated, separated by SDS-PAGE, transferred to a membrane, and autoradiograhed. The appearance of detectable bands on the autoradiograph indicates that the substrate has been phosphorylated. Phosphorylation may also be detected indirectly and in the absence of radioactivity, for example by using antibodies specific for the phosphorylated domain.

[0027] In the present method, the assay is performed in the presence and absence of the candidate agent. A detectable difference in phosphorylation in the presence of the agent is defined as any difference that is detectable by standard methods of assaying for phosphorylation, such as the *in vitro* kinase assay described above. Although the difference need not be quantitated, in a preferred embodiment the difference is at least about 10%.

[0028] It has been discovered in accordance with the present invention that phosphorylation of Foxa-2 leads to nuclear exclusion of Foxa-2 and inhibition of target genes involved in gluconeogenesis. Accordingly, the present invention further provides a method for inhibiting gluconeogenesis comprising contacting a cell capable of undergoing gluconeogensis with an agent that activates the phosphorylation of Foxa-2. The invention further provides a method of treating type 2 diabetes comprising administering to a subject in need of such treatment a composition comprising an agent that activates the phosphorylation of Foxa-2. In another embodiment, the present invention provides a method for increasing gluconeogenesis

comprising contacting a cell capable of undergoing gluoconeogenesis with an agent that inhibits the phosphorylation of Foxa-2. The invention further provides a method of treating hypoglycemia comprising administering to a subject in need of such treatment an agent that inhibits the phosphorylation of Foxa-2.

[0029] Such agents may be identified by the screening method described above. In addition, agents that mediate the kinase activity of Akt are known in the art and disclosed e.g., by Datta et al. (1999) Genes. Dev. 13: 2905-2927.

[0030] Agents identified by the methods of the present invention are useful for the treatment of diseases that may be ameliorated by altering the transcriptional activity of Foxa-2. The present invention provides compositions comprising such agents. The compositions may further comprise a diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant.

[0031] The formulation of pharmaceutical compositions is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. Formulations for use in present invention must be stable under the conditions of manufacture and storage and must also be preserved against the contaminating action of microorganisms such as bacteria and fungi. Prevention against microorganism contamination can be achieved through the addition of various antibacterial and antifungal agents.

[0032] The pharmaceutical forms of the present agents suitable for administration include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. Typical carriers include a solvent or dispersion medium containing, for example, water buffered aqueous solutions (i.e., biocompatible buffers), ethanol, polyols such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants, or vegetable oils.

[0033] Sterilization can be accomplished by any art-recognized technique, including but not limited to filtration or addition of antibacterial or antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid or thimerosal. Further, isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions.

[0034] Production of sterile injectable solutions containing the subject agents is accomplished by incorporating these compounds in the required amount in the appropriate solvent with various ingredients enumerated above, as required, followed by sterilization, preferably filter sterilization. To obtain a sterile powder, the above solutions are vacuum-dried or freeze-dried as necessary.

[0035] The subject agents are thus compounded for convenient and effective administration in pharmaceutically effective amounts with a suitable pharmaceutically acceptable carrier and/or diluent in a therapeutically effective dose.

[0036] All references cited herein are incorporated herein in their entirety.

[0037] The following non-limiting examples serve to further illustrate the present invention.

Example 1

Materials and Methods

The following materials and methods were used in subsequent examples.

Materials. Insulin was from Sigma, Ly294002 and PD 98059 were from Calbiochem.

[0038] **Generation of Plasmids.** Expression vectors for Foxa-1 and Foxa-2 were generated by cloning the coding region of either rat Foxa-1 or rat Foxa-2 into pcDNA3 either with or without fusion to an N-terminal Flag/HA-tag. Mutants (T156A and R153K) were generated by PCR mutagenesis using the Quickchange protocol (Invitrogen). Expression vectors for HA-Akt1 (pCMV-HA-Akt) were generated by

cloning the coding region of human Akt1 into pcDNA3 fused to an N-terminal HA-tag. The vectors encoding the HA-tagged forms of constitutively active Akt (pCMV-HA-myrAkt) and inactive Akt (pCMV-HA-Akt1$_{K179A}$) are as described by Cross et al. (1995) Nature 378: 785-789. Bacterial expression vectors of Foxa-2 and Foxa-2$_{T156A}$ were generated by cloning the cDNA into pGEX-4T2 (Pharmacia).

[0039] **Cell Culture.** HepG2 and HEK/293 cells were maintained in DMEM supplemented with 4.5 g/l glucose, 10% fetal calf serum, 2 mM glutamine; 50 µg/ml gentamycin/ streptomycin in a humidified incubator at 5% CO_2.

[0040] **Transfections and Transactivation assay.** HepG2 cells were grown to 60-70% confluence in a 24-well dish and transfected with 50 ng of each reporter gene (p6xCdx-TkLuc or pPEPCK-Luc), pCMV-β-Gal as internal reference and the expression vectors for wildtype or mutant Foxa-1 and Foxa-2 and human Akt1/Akt2 using the transfection reagent Fugene6 (Roche). Cells were grown for 48 hours and luciferase activity was measured using the Luciferase Detection System (Promega). Luciferase was normalized for transfection efficiency by β-galactosidase activity.HEK/293 cells were grown to 80% confluence in a 100mm cell culture dish and transfected with 10 µg of each expression vector for mutant or wildtype Foxa-2 and human Akt1 or Akt2 using Lipofectamine 2000 (Invitrogen).

[0041] **Expression and Purification of Recombinant GST-Foxa-2.** BL21 *E. coli* cells were grown to an OD_{600} of 0.8 and protein expression was induced by addition of 0.4 mM IPTG. Cells were harvested by centrifugation and lysed in 10 mM Tris/HCl, pH 7.4, and 30 mM NaCl by sonication at 4°C. Soluble *E. coli* proteins were equilibrated to 10 mM Tris/HCl, pH 7.4, 30 mM NaCl and chromatographed on a Mono-Q column (1 x 5 cm, 2 ml/min) using an FPLC system (Pharmacia). GST-Foxa-2 and GST-Foxa-2$_{T156A}$ eluted at approximately 300 mM NaCl. Fractions containing GST-Foxa-2 were pooled and concentrated using the Centriprep system with 10 kDa cut off (Millipore). The concentrated solution was subjected to gel

filtration on Supprose 12 (1.8 x 60 cm, 0.4 ml/min) in 10 mM Tris/HCl, 250 mM NaCl at pH 7.4. Purity of the protein was determined by SDS-PAGE.

[0042] **Immunoprecipitation**. Foxa-2 was precipitated from cell lysates using polyclonal anti-Foxa-2 antibodies (Ruiz i Altaba et al. (1993) Mech. Dev. 44: 91-108) bound to gamma-bind-sepharose (Pharmacia) overnight at 4°C. Akt was precipitated from cell lysates using monoclonal anti-HA antibody (Sigma) bound to gamma-bind-sepharose (Pharmacia) for 2 hours at 4°C. After washing of the precipitate the proteins were eluted with SDS-loading buffer, separated by SDS-PAGE (12%), and analyzed by Western blotting using either monoclonal anti Foxa-2 antibody (1:4000) or polyclonal anti-HA antibody (1:2000) (Sigma) and respective secondary antibodies linked to horseradish peroxidase (Calbiochem). Proteins were visualized by chemoluminescence detection using the ECL system (NEN).

[0043] **In vitro kinase assay**. Phosphorylation of Foxa-2 was analyzed using an *in vitro* kinase assay. Akt was precipitated from 200 µg of total protein lysate from HEK/293 cells transfected with pCMV-HA-Akt or pCMV-HA-LCK-Akt using anti HA antibody (Sigma) bound to gamma-bind-sepharose (Pharmacia) for 2 hours at 4°C. Precipitates were washed 3 times with kinase buffer (25 mM MOPS pH 7.4, 25 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, supplemented with protease inhibitor cocktail (Roche)) and incubated with 5 µg of either purified Foxa-2 and Foxa-2_{T156A} or with GST-Akt (positive control) in the presence of 0.5 µCi γ-ATP for 15 min at 37°C. Proteins were eluted with SDS-loading buffer, separated by SDS-PAGE (13.5%), and analyzed by autoradiography. Equal loading was confirmed by analyzing the expression levels of Foxa-2 by Western blotting.

[0044] **Immunofluorescence microscopy**. Cells were fixed for 30 minutes at room temperature with 2% paraformaldehyde. For immunofluorescent detection of Foxa-1 or Foxa-2, fixed cells were incubated with respective polyclonal antibodies (1:100) (Ruiz i Altaba et al. (1993) Mech. Dev. 44: 91-108) overnight at 4°C. After washing, the cells were treated with anti rabbit IgG secondary antibody linked to

Alexa Fluor 488 (Molecular Probes). Immunofluorescent staining was visualized using laser- scanning micrsocopy.

[0045] **Electrophoretic mobility shift assay.** Whole cell extracts from transfected HEK/293 cells (20 µg) were incubated with a ^{32}P-labeled double-stranded oligonucleotide probe with the Foxa binding sites of the Igfbp-1 promoter (Allander et al. (1997) Endocrinology 138: 4291-4300). The reaction was performed in a mixture containing Hepes buffer (20 mM, pH 7.9), KCl (40 mM), $MgCl_2$ (1 mM), EGTA (0.1 mM), DTT (0.5 mM), 4% Ficoll and poly(dIdC) at RT for 15 min. Competition analysis was performed by incubating the cellular extracts and the probe with the unlabeled oligonucleotide for a consensus Foxa binding site. Supershift analysis was carried out by incubating the nuclear extracts with either anti-Foxa-1 or anti-Foxa-2 antibody. The reaction mixture was loaded on a 6% non-denaturing polyacrylamide gel in TBE buffer (0.023 M Tris-borate, 0.5 mM EDTA) and run at 4°C. Bands were visualized by autoradiography.

Example 2

Insulin dependant decrease of Foxa-2 is mediated by PI3-kinase-Akt

[0046] To analyze whether Foxa-2 activity is regulated by insulin, HepG2 cells were transfected with a Foxa-2 expression vector and either plasmid pPepck-Luc or p6xCdx-TkLuc that contain a 621 bp promoter fragment of the human PEPCK gene or six Foxa-2 binding sites of the Cdx-2 gene upstream of a minimal promoter and the luciferase gene, respectively. Cotransfection of Foxa-2 with both reporter constructs led to an approximately five-fold increase in activity compared to control transfection. Treatment of the cells with insulin (100 nM) for the duration of transfection significantly decreased Foxa-2 activity. Coincubation with the PI3-kinase inhibitor Ly294002 (10 µM) prevented insulin mediated decrease of Foxa-2 activity, while the MAPKK1 inhibitor PD98059 (10 µM) did not influence insulin regulation of Foxa-2 activity (Fig. 1A).

[0047] To determine whether insulin decreases endogenous Foxa-2 activity in HepG2 cells, cells were transfected with the reporter construct alone and Foxa-2 activity was measured after stimulation with insulin and PI3-kinase or MAPKK1 inhibitors. Insulin stimulation led to a dose-dependent decrease of Foxa-2 activity (80% decrease at 500 nM insulin). This decrease in activity was ablated when cells were coincubated with Ly294002 but not with PD98059 (Fig. 1B).

[0048] Levels of mRNA were analyzed to determine whether inhibition of Foxa-2 target gene expression by insulin is controlled at a transcriptional or posttranscriptional level. The mRNA levels of Pepck, G6pc and Igfbp-1 in primary hepatocytes that were cultured either in the presence or absence of insulin, PI3-kinase or MAPKK1 inhibitors were measured. The mRNA levels were significantly reduced in insulin or insulin/PD98059 treated hepatocytes but not in controls (no insulin) or insulin/Ly294002 treated cells (Fig.1C). The reduced expression could not be attributed to increased expression levels of Foxa-1, 2 and 3, since expression levels of these genes did not significantly change in insulin-treated cells (Fig.1C). These data support previous reports by O'Brien et al. (1995) Mol. Cell Biol. 15: 1747-1758, indicating that insulin can inhibit the expression of Foxa target genes in hepatocytes, and that the rapid insulin-mediated reduction in transcriptional activity is unlikely due to alterations in expression levels of Foxa-1-3.

[0049] The demonstration that insulin signaling leads to a PI3-kinase mediated decrease in Foxa-2 activity led to an investigation of whether downstream targets of PI3-kinase are involved in the modulation of Foxa-2 activity. One prominent downstream target of PI3-kinase, which has been shown to modulate target gene activity by phosphorylation, is Akt (Datta et al. (1999) Genes Dev. 13: 2905-2927). To assess the involvement of Akt in mediating the insulin dependent decrease in Foxa-2 activity, HepG2 cells were transfected with expression vectors Foxa-1 or Foxa-2 and expression vectors for Akt1/2 or an inactive form of Akt ($Akt1_{K179A}$). Cotransfection of Foxa-2 with Akt1 or Akt2 completely abolished Foxa-2 activity while expression of the inactive Akt_{K179A} protein had no effect (Fig. 1D).

[0050] The Foxa-2 primary structure was analyzed for potential Akt phosphorylation sites Alessi et al. (1996) FEBS Lett. 399: 333-338. A putative Akt tyrosine phosphorylation site (RRSYTH) was identified in the human Foxa-2 protein at position aa152-157 that was completely conserved between human, mouse, rat, chicken, *X.laevis, C.elegans and S. pombe*. No Akt phosphorylation consensus sequences were detected in either Foxa-1 or Foxa-3 (Fig. 2A).

[0051] To determine whether the identified site was responsible for the Akt mediated regulation of Foxa-2 phosphorylation, the following experiment was performed. Two different mutants of Foxa-2 were generated: Foxa-2_{T156A} which cannot be phosphorylated, and Foxa-2_{R153K}, a mutant that is unable to bind to Akt (Alessi et al. (1996) FEBS Lett. 399: 333-338). HepG2 cells were cotransfected with p6xCdx-TkLuc, expression vectors for either wt Foxa-2, Foxa-2_{T156A} or Foxa-2_{R153K}, together with pCMV-HA-Akt-1 or -2. A dose-dependent inhibition of Foxa-2 activity was observed when transfected with increasing amounts of the Akt1 or Akt2 expression vectors (Fig. 2B). This decrease was not observed in cotransfections with either Foxa-2_{T156A} or Foxa-2_{R153K}, indicating that the identified site is responsible for Akt-mediated regulation of Foxa-2 activity. The effect of wt and mutant Foxa-2 proteins on the activity of Pepck gene transcription was analyzed by coexpressing these proteins together with pPepck-Luc. Transcriptional activity of the Pepck promoter was increased about 6-fold in cells cotransfected with Foxa-2. A dose-dependant decrease was observed when increasing amounts of Akt1 or Akt2 expression vectors were cotransfected. No decrease in activity was observed in transfection with either Foxa-2_{T156A} or Foxa-2_{R153K} using increasing amounts of Akt (Fig. 2C).

Example 3

Akt interacts with and phosphorylates Foxa-2 at positionT156

[0052] To demonstrate that Akt modulates Foxa-2 activity by direct interaction with the putative Akt phosphorylation site, immunocoprecipitation experiments were

performed. HEK/293 cells were transfected with Foxa-2, Foxa-2_{T156A}, or Foxa-2_{R153K} and HA-Akt. Foxa-2 was precipitated using a polyclonal anti-Foxa-2 antibody, HA-Akt was precipitated with a monoclonal anti-HA antibody. The precipitates were separated by SDS-PAGE and analyzed by Western blotting. As can be seen in Fig. 3 precipitation of HA-Akt led to coprecipitation of Foxa-2 and Foxa-2_{T156A} but not of Foxa-2_{R153K}. Comparison of Foxa-2 and Foxa-2_{T156A} showed that interaction of Foxa-2 with Akt is approximately 2-fold weaker than wildtype Foxa-2. Conversely, precipitation of Foxa-2 yielded similar results, as Akt could be coprecipitated together with Foxa-2 and Foxa-2_{T156A} but not with Foxa-2_{R153K}. No differences in interaction of Foxa-2 and Foxa-2_{T156A} could be seen using this approach (Fig. 3).

[0053] To confirm that Foxa-2 can be phosphorylated by Akt kinase activity, the following experiment was performed. Recombinant GST-Foxa-2 and GST-Foxa-2_{T156A} proteins were expressed in *E.coli* BL21 cells and the soluble protein was purified by anion-exchange chromatography and subsequent size exclusion chromatography. Active Akt was purified from transfected HEK/293 cells by immunoprecipitation with anti HA-antibody. GST-Foxa-2, GST-Foxa-2_{T156A} or GST-Akt (GST fused to a consensus Akt phosphorylation site) (Vandromme et al. (2001) J. Biol. Chem. 276: 8173-8179) as positive controls were coincubated with either control cell precipitates (untransfected cells), precipitated HA-Akt or precipitated constitutive active Akt in the presence of 0.5 μCi [γ^{32P}]-ATP. Figure 4 shows that wildtype Foxa-2 was phosphorylated by either Akt or myrAkt, while no phosphorylation could be observed using Foxa-2_{T156A} protein as substrate. Equal loading of wildtype Foxa-2 and Foxa-2_{T156A} was demonstrated by Western blotting of the phosphorylation reactions (Fig.4).

Example 4

Foxa-2 phosphorylation by insulin–PI3-kinase–Akt signaling leads to nuclear export

[0054] The mechanisms underlying the inhibitory effect of Foxa-2 phosphorylation on the transcriptional activation of target genes were examined. Mechanisms that may account for the inhibitory effects of Akt on Foxa-2 function include an Akt induced reduction of total Foxa-2 expression levels, impairment of binding to DNA, impairment of Foxa-2's intrinsic transcriptional activation or repressor function or by changes in Foxa-2's nuclear localization. It was found that expression of Akt did not significantly change mRNA or protein expression levels of Foxa-2 in HepG2 cells. The DNA binding activity of nuclear extracts from HepG2 cells transfected with either wildtype or Foxa-2_{T156A} expression vectors was compared. Electrophoretic mobility shift assays were performed to investigate if mutant Foxa-2 proteins can bind to a Foxa binding site of the Igfpb-1 promoter (Allander et al. (1997) Endocrinology 138: 4291-4300). Wildtype and phosphorylation deficient mutant Foxa-2_{T156A} bound equally to ^{32}P-labelled oligonucleotide probes that contained the Foxa binding site. These data indicate that the phosphorylation state of Foxa-2 does not lead to impairment in DNA binding (Fig. 5A).

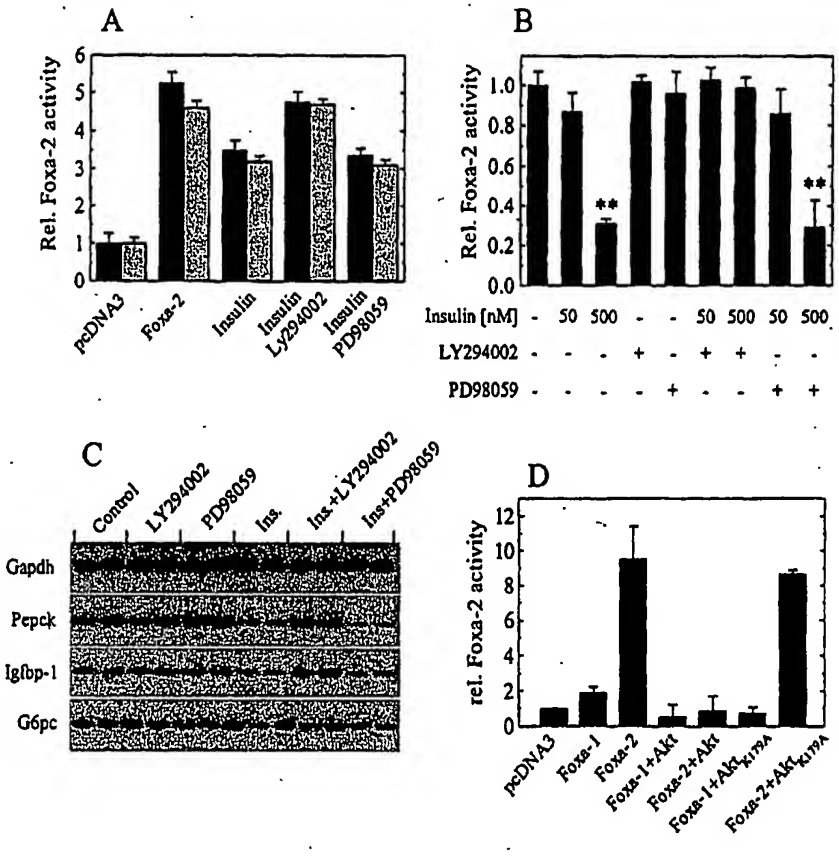
[0055] To determine whether Akt-induced phosporylation of Foxa-2 has an effect on the subcellular distribution of this transcription factor, the following experiment was performed. HepG2 cells were grown to 60% confluency and endogenous Foxa-1 and Foxa-2 proteins were visualized by immunofluorescence after staining with anti Foxa-1 and Foxa-2 antibodies. Cells were either examined in the absence or presence of insulin (50 nM) and/or Ly294002 or PD98059, and after transfection with either Akt1 or Akt2 expression vectors (Fig. 5B). When the endogenous PI3-kinase–Akt pathway was inhibited (by serum starvation (Control) or treatment with Ly294002 in the presence of insulin) the endogenous Foxa-2 protein was localized almost exclusively in the nucleus. In contrast, cells in which the PI3-kinase–Akt pathway was activated by either treatment with insulin or by overexpression of Akt1/2, Foxa-2 was efficiently excluded from the nucleus and largely detected in the cytoplasm (Fig. 5B). Treatment of cells with MAPKK1 inhibitor PD98059 had no effect on insulin stimulated nuclear exclusion of Foxa-2.

The subcellular distribution of Foxa-2 in cells that were stimulated with insulin and expressed a dominant negative form of Akt (Akt_{K179A}) were resistant to nuclear exclusion. In contrast to the drastic changes in subcellular localization of Foxa-2 upon stimulation of the PI3-kinase–Akt pathway, Foxa-1 protein was not responsive to the activation of this pathway. The same results were obtained when using a glucose responsive pancreatic β-cell line (Min6). To examine if the effect of Akt on the subcellular localization of Foxa-2 was due to the phosporylation of T156 residue, wt and mutant Foxa-2_{T156A} HA-tagged protein were expressed in HepG2 cells. The intracellular distribution of this protein in the presence and absence of insulin was examined. In contrast to the wildtype Foxa-2 protein, Foxa-2_{T156A} was exclusively localized in the nucleus after activation of the PI3-kinase–Akt pathway with insulin (Fig. 5C). Together, these data demonstrate that insulin stimulation induces phosphorylation via the endogenous PI3-kinase–Akt pathway of a conserved residue, specific for Foxa-2, and that this site plays a crucial role in sequestering Foxa-2 in the cytoplasm, thereby inhibiting Foxa-2's ability to activate transcription of target genes in the nucleus.

ABSTRACT

Methods of identifying agents that mediate the phosphorylation of the transcription factor Foxa-2 are provided. Such agents are useful in methods of inhibiting gluconeogenesis and in treating Type 2 diabetes.

Figs. 1A-D


A
Rel. Foxa-2 activity
pcDNA3
Foxa-2
Insulin
Insulin LY294002
Insulin PD98059
B
Rel. Foxa-2 activity
Insulin [nM]
LY294002
PD98059
C
Control
LY294002
PD98059
Ins.
Ins.+LY294002
Ins+PD98059
Gapdh
Pepck
Igfbp-1
G6pc
D
rel. Foxa-2 activity
pcDNA3
Foxa-1
Foxa-2
Foxa-1+Akt
Foxa-2+Akt
Foxa-1+Akt$_{K179A}$
Foxa-2+Akt$_{K179A}$

Figs. 2A-C

A

	147	196
human FOXA-2	DPKTYRRSYT HAKPPYSYIS LITMAIQQSP NKMLTLSEIY QWIMDLFPFY	
mouse Foxa-2	DPKTYRRSYT HAKPPYSYIS LITMAIQQSP NKMLTLSEIY QWIMDLFPFY	
rat Foxa-2	DPKTYRRSYT HAKPPYSYIS LITMAIQQSP NKMLTLSEIY QWIMDLFPFY	
chicken Foxa-2	DPKTYRRSYT HAKPPYSYIS LITMAIQQSP NKMLTLSEIY QWIMDLFPF.	
X.laevis Foxa-2	DPKTYRRSYT HAKPPYSYIS LITMAIQQSP NKMLTLSEIY QWIMDLFPFY	
D. melanogaster	DPKTYRRSYT HAKPPYSYIS LITMAIQNNP TRMLTLSEIY QFIMDLFPFY	
human FOXA-1	DAKTFKRSYP HAKPPYSYIS LITMAIQRAP SKMLTLSEIY QWIMDLFPYY	
mouse Foxa-1	DAKTFKRSYP HAKPPYSYIS LITMAIQQAP SKMLTLSEIY QWIMDLFPYY	
human FOXA-3	MPKGYRAP.A HAKPPYSYIS LITMAIQQAP GKVLTLSEIY QWIMDLFPYY	
mouse Foxa-3	MAKGYRRPLA HAKPPYSYIS LITMAIQQAP GKMLTLSEIY QWIMDLFPYY	

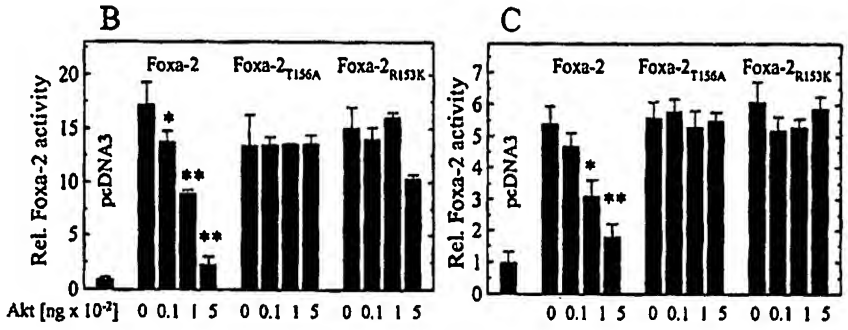


Fig. 3

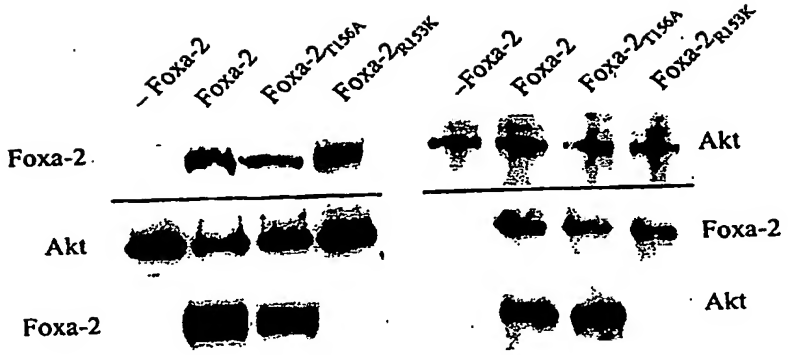
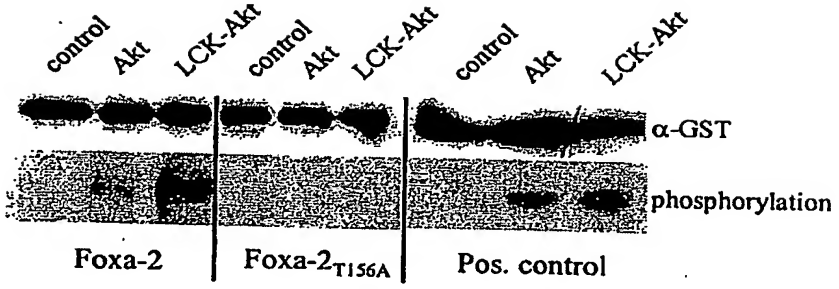

-Foxa-2
Foxa-2
Foxa-2T156A
Foxa-2R153K
-Foxa-2
Foxa-2
Foxa-2T156A
Foxa-2R153K
Foxa-2
Akt
Akt
Foxa-2
Foxa-2
Akt

Fig. 4


control
Akt
LCK-Akt
control
Akt
LCK-Akt
control
Akt
LCK-Akt
α-GST
phosphorylation
Foxa-2
Foxa-2T156A
Pos. control

Figs. 5A-C